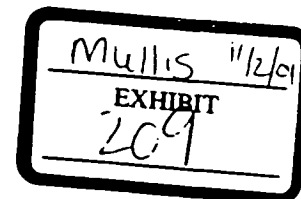# The Polymerase Chain Reaction in an Anemic Mode: How to Avoid Cold Oligodeoxyribonuclear Fusion

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EXHIBIT
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There is a very simple relationship between the number of molecules of polymerase in a PCR and the number of primed templates that can be extended in a given cycle of that reaction. The ratio is one to one. None of the conventions currently in use for describing the quantitative aspects of biochemical reactions are very useful in illuminating such stoichiometrical relationships. Before turning to the spectre of cold oligodeoxyribonuclear fusion reactions, we would like to discuss this situation and propose a minor change.

Enzymes have been traded and generally utilized by the activity unit for about 100 years, a unit of DNA polymerase, for example, being the amount required to convert under certain conditions 10 nmoles of deoxyribonucleotides into an acid-insoluble form in 30 min: not a very useful definition nor one that conveys any useful information to the molecular biologist except price. Oligodeoxyribonucleotides are traded by the OD_{260} unit, which was defined out of necessity as that amount of an oligomer that when dissolved in 1 ml will show an optical density of 1.0 at 260 nm across a 1-cm pathlength. Other nucleic acids are described by weight, even when their molecular weights are known; and magnesium, buffers, and dNTPs mysteriously are described by the fractional part that their count represents of Avogadro's number, even though this may be a minuscule fraction. Although all these units can be converted to a common

currency, they do not do so in one's mind automatically, and consequently a great deal of clarity is sacrificed in our communications with each other and with our own experimental results.

Perhaps the worst of the lot in terms of the number of errors it causes in day-to-day calculations is the system based on Avogadro's number. For here we must deal with infinitesimal fractions of preposterous amounts. We do not buy gasoline by the cubic mile for obvious reasons. Avogadro's number is not an intrinsic chemical constant nor is it suitable for biochemistry in the twentieth century. It is derived from a measurement of the size of the Earth heroically made by the French in the eighteenth century. The number depends on the Gram which came from the Cubic Centimeter full of water at 4°C, which derived from the Meter, which had been defined as one ten-millionth of the distance from the North Pole to the Equator along a line passing between the Nôtre-Dame and the guy selling chestnuts there in the winter of 1799. We should not feel obliged to use Avogadro's number unless it is really useful.

In this paper we will try not talking about things to the twenty-third power, and particularly will try to avoid saying things like "One haploid human cell should contain 1.5×10^{-24} moles of any unique sequence." If there was any sense at all in installing the gram-molecular-weight of something as a unit of measure, then it was expressly to avoid the need for dealing

with huge exponents, and was not introduced as providing a cryptic way of expressing the concept "one."

We will refer to molecules as if they were things, one or more at a time; if we need to refer to concentration, we will say how many molecules there are in a given volume. And since volumes in biochemistry are conveniently measured in microliters, we will use the microliter. This system has the advantage of clarity.

Thus, the amount of something, be it a protein, a nucleic acid, or a magnesium atom, will be described in terms of how many of them there are. And while we are simplifying, we will drop the annoying convention of superscripts and just say 4E12, for instance, if we mean we have 4 times 10 to the 12th power of something. Neither will we lapse into Latin text unless begged by the specific occasion. So one thing is 1, two things are 2, a thousand is E3. There are approximately E4 copies of the human genome in a microliter of human blood. This concentration will be written U4, meaning ten to the fourth per microliter. Millimolar, which we will want to dispense with directly as a remnant of an ancient measuring system, we will for the time being translate as 6U14. This requires that we drop the .023, but we don't know anyone who pipettes that well anyhow.

One of our PCR protocols looks like this: Note that the two columns are not describing concentrations that are truly equal; we have modified the actual concentrations slightly so as to

conform more roundly to the new sys-
tem. It is our experience that small
...ges in parameters like concentra-
... in PCR do not matter.

	After	Before
Tris HCl	50U15	80 mM
Ammonium sulfate	10U15	20 mM
Magnesium chloride	5U15	10 mM
dNTPs	5U14	1 mM
Oligonucleotides	5U11	1 μM
Taq polymerase	3U9	5 units
DNA, human	3U3	1 μg

Volume 100 μl
Cycles: alternately 65°C, 30 sec; 98°C,
30 sec.

THE POLYMERASE CHAIN REACTION

In a PCR where we are trying to double
the number of molecules representing
a particular target sequence every
cycle, we need enough oligonucleo-
tides to prime all the appropriate se-
quences, and to do it before these
rehybridize to themselves; and we need
enough enzyme to engage each primed
sequence; and then enough dNTPs to
furnish the raw materials of polymer-
ization. The enzyme, being the reagent
...ensed by biochemical supply
...ses in the lowest concentration
(3U10 to 3U11) almost always becomes
limiting before any of the others. En-
zyme limitation leads to something we
call "anemic cycles." More will be said
later of these and of the attendant cold
deoxyribonuclear fusion reactions that
they promote.

A typical PCR might begin with 3E5
copies of human DNA and the alleged
purpose of amplifying a single-copy
human gene. In an indisputable
ethidium bromide gel band, there are
about 5E12 base pairs of DNA. That
means, by way of an example, that for
a 100-bp amplicon (I didn't make up
this word; I heard it at a meeting but I
like it) we need 5E11 copies of the 100-
bp amplicon in a 100-μl reaction
volume to have 5E12 base pairs in a
10-μl aliquot for the gel. So we need
some number of cycles to amplify the
3E5 copies to 5E11. How many?

A basic equation that describes PCR
is an old one which describes a lot of
things that grow by division:

$$Y_n = (1 + e)^n$$

...ere Y_n is the amplification factor

after n cycles at an efficiency of e,
which is a number from 0 to 1 that
refers to that fraction of sequences
available for duplication in each cycle
that actually got duplicated.

So how many cycles do we need?
We take the natural logarithm and
rearrange the formula:

$$n = \ln Y_n / \ln (1 + e)$$

Y_n in this case stands for the ratio of
how many molecules we want to how
many molecules we started with, and
including the 10-fold aliquot factor we
have

$$Y_n = 5E11/3E5 = 1.76E6$$

and if we say optimistically that $e = 1$
then

$$n = \ln 1.76E6/\ln 2 = 20.7 \text{ cycles.}$$

So in 21 cycles we should be able to
start with 3E5 copies of human DNA
and get a band on an ethidium
bromide gel from a one-tenth aliquot.
Sometimes we do and sometimes we
don't.

In the last cycle, assuming every-
thing has worked perfectly, we would
need to be making 2.5E11 molecules,
which requires 2.5E11 molecules of
Taq translating back to about 10 units.
This requirement is independent and
in spite of the specific activity of the
enzyme. The specific activity is mea-
sured as initial velocity of polymeriza-
tion on a long template such as M13;
and what we are dealing with in PCR is
not simply that, but rather the ability
to synthesize many separate molecules.
And here we must take into account,
we think, the strong affinity which the
thermophilic polymerases have for
their substrates. Thus, the polymerases
are disinclined to ever come loose from
a DNA molecule, even when they have
reached the end of it. Coming to the
end of a template probably has no evo-
lutionary precedence for them because
the process of repair and replication in
bacteria never involves the, perhaps
shocking and at least novel, experience
for a polymerase of reaching the end of
a template. Bacterial genomes are
round, and bacterial polymerases are
unlikely to be any better equipped to
jump off the end of a linear PCR
amplicon than are tightrope walkers to
walk off the end of a rope. (If this is
certainly true then the denaturation
step in PCR serves a double purpose,

but this is another concept best left for
now.) What is true is that when the
number of primed templates exceeds
the number of *Taq* molecules, some of
the primed templates do not get ex-
tended. This is what we refer to as an
anemic cycle.

Anemia will occur at an earlier or
later cycle in our reaction depending
on how many enzyme molecules we
have employed and, very critically, on
how many nontarget amplicons we
have inadvertently created, and are
amplifying along with the intended
target. Nontarget amplicons can arise
from a number of factors, but most fre-
quently they are generated by one or
more extension cycles being carried
out under nonstringent priming condi-
tions, i.e., low temperature.

If when anemia occurs the in-
tended target is a predominant species
in the reaction, then we can simply do
extra cycles until we slowly build up
the amount of target amplicon desired.
For the amplification of human genes
and especially of less complex targets,
this is often close to being the case,
which is why PCR generally works
without a hitch for genomic targets,
even though a considerable part of the
amplification occurs under anemic
conditions.

This is not the case, however, if one
is trying to amplify something like a
single molecule of a foreign DNA in
the presence of 3E5 copies of a com-
plex host DNA, like, for example, HIV
from 30 μl of human blood. Here the
problem arises that when the reaction
goes anemic, there are in the reaction
mixture the requisite 3E10 fragments
of primed DNA per unit of *Taq*, which
is one molecular of primed DNA per
molecule of *Taq*, and which is why the
reaction is becoming anemic, but hard-
ly any of these molecules correspond
to the intended target. If, as is typical
of most PCR protocols, anemia begins
around cycle 15, the target, which has
been copied with 100% efficiency up
till now, is present in only 5E4 copies
and at the very low reaction efficien-
cies that rapidly occur within a few
cycles of the appearance of anemia; the
target is not likely to ever reach 5E11
copies and easy visibility on ethidium
bromide gels. In fact, with 30 addi-
tional anemic cycles at yields around
20%, the target may be amplified up

another

$$Y_n = (1.2)^{30} = 237\text{-fold}$$

to 1.2E7 copies, where it might be detected with ^{32}P or recent methods employing enzyme linkage.

But it is also possible that amplification of the target might come to an almost complete halt due to the fact that: Once the state of anemia is reached not all amplicons will be subject to the same degree of further amplification, and it may not even be amplified 237-fold and may not be detected at all. For in anemia there is a shortage of resources in the reaction and only those fragments that compete best for them will be duplicated. The factors that govern this competition are not complicated and weigh heavily on the side of short fragments, and with this we arrive at the spectre of cold deoxyribonuclear fusion and the short ugly fragments to which it gives rise.

COLD DEOXYRIBONUCLEAR FUSION

From empirical observations no one will doubt that, once established, shorter fragments in an anemic reaction will be amplified preferentially. Little molecules seeming to cluster in the region on the gel that would be about two primers long if primers were double stranded seem to pop up out of nowhere and dominate the amplification. Something seems to be catalyzing the fusion of oligodeoxyribonucleotides. More cycles lead to more intensity of these little fragments but not much more of your target fragment. Their size has earned them the misnomer of "primer dimers," in spite of the fact that sequences have always revealed non-primer-derived core regions, which the concept of "dimer" does not imply. A bold solution to these "ugly little fragments" in the case that they do not interfere with the detection of one's intended amplicon was publicly announced by Richard Gibbs (in a talk at the Keystone UCLA Symposium on PCR in 1989). "Run them off the bottom of the gel."

As uplifting as this solution might have been to the people who have suffered from the "ugly little fragments," it is of no help in those cases where the appearance of these PCR artifacts interferes seriously with the amplification

and detection of the intended target. What we are addressing here is the difference between the amplification of a genomic target (say 3E5 up to 5E11, or a little more than a million-fold) where the background amplicons are a nuisance that can be run off the gel and the amplification of a needle in a haystack (one copy up to 5E11 or 100-billion-fold) where the nonintended background amplifications can prevent the intended target from ever showing up on the gel.

The more complex the background and the longer the period when robust amplification is occurring, the more likely will be the occurrence of a very efficiently amplified short fragment. When the reaction becomes anemic, this fragment will be selectively amplified further. It is a molecular scale model of evolution (especially since medium-sized amplicons as they accumulate can increasingly give rise to shorter amplicons by internal mispriming). Notice that both primers are required, rather than two copies of one. The amplicon produced from two copies of one primer would have internal hairpin symmetry in each single strand and would be amplified at a very low efficiency.

Theoretical considerations indicate that the inverse size dependence of amplification efficiency is a fairly steep one. The question becomes, then, in a practical sense, how to prevent the initial generation of these unwanted little fragments in what was hopefully to be a high-gain amplification of a usefully large fragment. Although it should be noted that when there is a choice in regard to the size of the intended amplicon, it certainly makes no sense, *ceteris paribus*, to design it larger than necessary.

One way to prevent short ugly fragments from overwhelming a reaction is to prevent the extension reactions from beginning until the temperature is high enough to insure that very few nonperfect-match primings get extended. The generation of very short fragments is inversely dependent on the stringency of priming and extension.

When a reaction mixture similar to that described earlier using heat-denatured human DNA was subjected to 5 min at room temperature, about 1

ng of new DNA was produced. This was not in any way associated with target amplification, as the primers were designed to amplify a sequence from the *gag* region of HIV and do not specifically amplify any human sequence. This was scattered background primer extension that occurred from the human DNA, and was detected as precipitable counts or a broad smear on a gel centered around 500 bp when one of the dNTPs was α-labeled. Somewhat surprisingly, this initial 5 min. during which only 0.1% of new DNA was synthesized, had a devastating effect on subsequent amplification efficiency compared to a reaction where no primer extensions were allowed to proceed below 65°C.

The powerful effect of the newly synthesized nanogram of DNA is due to the fact that each molecule begins with one of the primers. For purposes of the following discussion, we will describe these fragments as "half-amplicons." Now the probability of producing new DNA comprising full amplicons by two cycles of random priming, where in the second cycle priming on a half-amplicon generates a fragment with two perfect primer sites, will be given by an expression involving the square of the probability for producing half-amplicons. Thus, the probability for generating unwanted PCR amplicon DNA by repeating the 5-min, low-stringency cycle to a first approximation would be

$$P \times P = (1\text{ ng}/1\text{ μg}) \times (1\text{ ng}/1\text{ μg}) = E - 6$$

Assuming that the full amplicons would on average be half the size of the half-amplicons, the number of amplicons generated would be

$$N = (E - 6)(1.8E15)/250$$
$$N = 7.2E6$$

where 1.8E15 is the number of nucleotides in 1 μg of DNA.

But in our experiment after subjecting the reaction mixture to one low-stringency cycle for 5 min, we entered a normal PCR cycle where extension took place at 65°C. We do not know the actual amount of new amplicon DNA that was synthesized in the second cycle, but it is instructive, in terms of understanding the influence that one low-temperature cycle can have on a PCR reaction, to examine a range of

probabilities and examine the outcome for each. Below we have compared the number of amplicons of average size 250 nucleotides that would be produced in two cycles, where in the one case, A, both cycles were done under a stringency that would produce new DNA with a probability of P, and where in the other case, B, the first cycle was done under low-stringency conditions in which new DNA was produced with a probability $E - 3$. The first two columns list the number of amplicons produced and the second two columns predict the number of cycles that will be required for those amplicons to give rise to an anemic reaction.

AMPLICONS GENERATED AT CYCLE TWO UNDER CONDITIONS OF INCREASING STRINGENCY IN CASES A AND B

P	A	B	A	B
$E-4$	1.4E5	1.4E6	21	17
$E-5$	1.4E3	1.4E5	27	21
$E-6$	14	1.4E4	34	24
$E-7$	.14	1.4E3	41	27
$E-8$	0	140		30
$E-9$	0	14		34

In case B corresponding to an amplification in which a cold cycle was performed, either intentionally or inadvertently, even if a very stringent routine were followed after the first cold cycle ($E - 8$, for instance), it would be very unlikely if a single target molecule could be amplified and readily detected in the presence of over 100 other amplicons. In this case, the reaction would be anemic after a minimum of only 30 more cycles, at which point a single molecule would have been amplified up to about 4E9, which would not yet be visible on an ethidium gel.

The statistical manipulations here have been very much first approximations, but the conclusions drawn are in accord with experimental results. In the experiment referred to above (K. Mullis, F. Ferre, and F. Faloona, in prep.), we were able to achieve the required sensitivity to detect one molecule of HIV DNA in the presence of 1 μg (3E5 copies) of human DNA on an ethidium-stained gel. This represents a 1000-fold increase in sensitivity compared to the same protocol after a 5-min exposure of the complete reaction mixture to room temperature. Starting the reaction cold with what we considered to be native DNA lowered the detection limits about 100-fold under our conditions. It is our opinion, but we have not demonstrated this, that thorough denaturation of chromosomally integrated targets is necessary to insure quantitatively reproducible amplifications, especially when the number of starting sequences is close to one. This requires boiling for several minutes and cannot be done in the presence of enzyme. The simplest way to avoid cold oligodeoxyribonuclear fusion and its consequent ugly little fragments is to put the reaction together with the DNA denatured, but without the dNTPs, until the temperature is brought up to about 80°C. At this point, with the tubes in a heat block ready to go into a machine that is holding at 80°C, the dNTPs can be added to a reactionally inactive mixture, taken up to 98°C for 30 sec, and then all samples will enter the PCR cycles equivalently; and uniformity and high-gain amplifications can be expected and accomplished.

An awareness of the nature of anemia in PCRs, its potentially catastrophic results, and ways of preventing it, only one of which has been mentioned here, might be beneficial to many workers who have experienced problems with PCR. Two other examples that come to mind where an abundance of unwanted amplicons might lead to a failure of the desired amplification are amplification from RNA where a low-stringency reverse transcriptase step is interjected and experiments in the clever but as yet to be extensively applied technique of inverse PCR.

PCR

METHODS ● AND ● APPLICATIONS

The Ligase Chain Reaction

DNA Polymerase Fidelity

Vectorette PCR and Genomic Walking

Determining the 5′ End Sequence of mRNAs

Self-sustained Sequence Replication

CSHL
PRESS